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Inhibitory Effect of Dietary Soybean Meal on the Establishment of a *Clostridium* Strain in the Gastrointestinal Tracts of Mice

CLAUDE LHUILLERY,[1]* YVES DEMARNE,[1] ROBERT DUCLUZEAU,[2] AND ANTONIO CLARA[2]

Station de Recherches de Nutrition[1] and Laboratoire d'Ecologie Microbienne,[2] Institut National de la Recherche Agronomique–Centre National de Recherches Zootechniques, 78350 Jouy-en-Josas, France

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The presence of soybean meal in the diet of gnotobiotic mice was shown to prevent the establishment of a clostridial strain in the gastrointestinal tract. Soybean meal did not inhibit the growth of *Clostridium* in vitro, suggesting that the host plays a role in the observed inhibition of bacterial growth. The inhibitory factor in the soybean meal contains at least two components.

The biological succession which occurs in the gastrointestinal tract of conventional mammals during the first weeks of life is well described (15). Little is known, however, about the factors which regulate this succession. The host's diet may be one of the factors which influence microbial populations in the gastrointestinal tract. Studies have shown that alterations in milk composition result in alterations of the microflora in animals (3, 8, 11) and in babies (1, 13, 18). Unfortunately, although various hypotheses have been made (2, 5, 9, 10, 13, 14), the precise mechanisms by which diet influences the establishment of the gastrointestinal microflora are not known.

In a previous paper (6), we had shown that the establishment of a bacterial strain (*Clostridium* En.) in the gastrointestinal tract of gnotobiotic mice was directly dependent on the composition of the diet eaten by the animals. When germfree mice were fed certain diets, a per os inoculation of the clostridial strain resulted in a rapid and stable colonization of the gastrointestinal tract. These diets were therefore called "permissive." On the other hand, the strain could not establish when the mice were fed "nonpermissive" diets.

The work presented here deals with the characterization of the active component of the diets and some hypotheses concerning the mechanisms by which diet may regulate the establishment of bacteria in the gastrointestinal tract.

MATERIALS AND METHODS

General procedure. Adult germfree C3H mice were maintained in plastic isolators fitted with a rapid transfer system (15). Diet and water were fed ad libitum.

Unless otherwise stated, the experimental procedure was as follows. Diets were prepared shortly before the experiment and sterilized by 4-Mrad γ-irradiation. Mice were housed in groups of two or three, and each group was fed a different diet. Each diet was given to the animals a week before oral inoculation with the clostridial strain and throughout the experiment. The germfee state of the animals was verified for each group during the week of adaptation to the dietary treatment. The number of clostridia in the feces of the animals was determined at intervals during the 2 weeks after inoculation. The feces of each group of animals were pooled for examination. Details about the *Clostridium* strain, culture media, inoculation, and microbial methods have been described previously (6).

In each experiment, two kinds of responses were possible: either the *Clostrodium* En. became established in the gastrointestinal tract of mice at a dominant level (i.e., $>10^7$ bacteria/g of fresh feces) in less than 48 h after inoculation and remained at that level throughout the experiment; or it did not become established and could not be detected in the feces after inoculation ($<10^2$ bacteria/g). In the first case, the dietary treatment was called "permissive"; in the second, it was called "nonpermissive."

Diets. Only one commercial diet was used in these experiments: diet U (UAR D03), composed of (in grams per kilogram): 625 g of cereals, fat, and carbohydrate; 100 g of vegetable proteins (soybean meal and yeast); 235 g of animal proteins (fish and milk casein); and 40 g of mineral and vitamin mix.

The other diets were prepared in the laboratory. The two basic diets have been described previously: the nonpermissive diet A (4) and the permissive diet PG (7). Table 1 gives the diet compositions.

RESULTS

Active fraction of the diets. Possible explanations for the different effects of the diets on the establishment of the clostridial strain were: (i) there was a growth factor in the permissive diets; or (ii) there was an inhibitory factor in the nonpermissive diets. To solve this problem and to determine the active fraction, diets of intermediate composition were prepared by replacing one component (e.g., lipid or protein source) of

TABLE 1. Effect of dietary composition on the establishment of *Clostridium* in the intestines of mice

Diet	Composition (wt %)									Establishment
	Ground corn	Soybean meal	Skim milk powder	Corn oil	Tallow	Cellulose	Cerelose	Minerals + vitamins		Amino acid supplement	
								Mixture 1	Mixture 2		
A (non-permissive)	51	30		10		3		5		1	No
PG (permissive)			66		25		5		4		Yes
C			81	10		3		5		1	Yes
D	41.5	24.5			25		5		4		No
E	51	30		10		4			4	1	No
F			66		25		4	5			Yes
G			81	10		4			4	1	Yes
H	41.5	24.5			25		4	5			No

the permissive diet PG by a corresponding component of the nonpermissive diet A. All diets were sterilized by irradiation and tested in vivo as described above.

In the first experiments, skim milk powder was replaced by soybean meal plus ground corn. Six intermediate diets (C, D, E, F, G, and H) were prepared.

All diets containing soybean meal plus ground corn (diets A, D, E, and H) were nonpermissive, and all diets containing milk powder (diets PG, C, F, and G) were permissive (Table 1). All other dietary components except soybean meal, ground corn, or milk powder were present in both the permissive and nonpermissive diets (see, for example, diet E and diet G) and therefore could not be involved in the inhibitory effect.

Three new diets were now prepared based on the permissive diet PG. In these diets, skim milk powder was replaced by its two major constituents (i.e., 50% lactose and 40% casein), and the two components were combined either with soybean meal or with ground corn.

In diet 1, casein and lactose were present in the same proportions as in diet PG. Diet 2 contained a combination of casein and ground corn, and diet 3 contained lactose and soybean meal (Table 2). *Clostridium* En. did not establish in the gastrointestinal tract of mice when the animals were fed diet 3 (lactose plus soybean meal). On the contrary, the clostridial strain rapidly colonized the tract of mice fed the other two diets.

The only other possible combination (soybean meal associated with casein) was present in the commercial diet U (see Materials and Methods). This diet, when tested, proved to be nonpermissive (6).

From these experiments, we concluded that the inhibitory factor which prevented the establishment of *Clostridium* En. was contained in the soybean meal. Further experiments were then undertaken to more precisely characterize the inhibitory factor of soybean meal and to understand the mechanism of inhibition.

Minimal inhibitory proportion of soybean meal in the diet. All of the nonpermissive, semisynthetic diets used above contained at least 30% soybean meal. To determine the minimal inhibitory proportion, diets containing, respectively, 2, 5, and 20% soybean meal were prepared from the permissive diet PG by replacing an increasing quantity of skim milk by the same quantity of soybean meal. The diets, soybean 2, soybean 5, and soybean 20, were sterilized and fed to germfree mice to test their influence on the establishment of *Clostridium* En. (Table 3).

All diets containing at least 5% soybean meal inhibited the establishment of *Clostridium* En. in

TABLE 2. Importance of dietary soybean meal in inhibiting the establishment of *Clostridium* in the intestines of mice

Diet	Composition (wt %)							Establishment
	Casein	Lactose	Soybean meal	Ground corn	Tallow	Cerelose	Minerals + vitamins (mixture 2)	
PG (permissive)	66				25	5	4	Yes
1	33	33			25	5	4	Yes
2	25			40	25	5	4	Yes
3		33	33		25	5	4	No

the gastrointestinal tract of mice. Only the soybean 2 diet was permissive. We concluded that at least 5% soybean meal in the diet was necessary to exert the inhibitory effect.

Soybean meal fractionation. To determine the nature of the inhibitory component of soybean meal, the following fractionation procedure (Fig. 1) was used. Forty grams of soybean meal was suspended in 800 ml of distilled water and gently stirred for 1 h. The suspension was then centrifuged at 20,000 × *g* for 15 min. Pellet I, thus obtained, was extracted a second time in the same way. After the second centrifugation, supernatants I and II were pooled (= fraction S) and freeze-dried. Pellet II (= fraction P) was suspended in a small volume of distilled water and freeze-dried.

The soybean meal fractions were mixed in appropriate porportions with permissive diet PG. Since a minimal proportion of 5% soybean meal in the diet was required to exert the inhibitory effect, proportions were calculated on the basis of 10% soybean meal in the diet to offset possible losses occurring during the extraction procedure. Fraction S, corresponding to the initial 40 g of soybean meal, was mixed with 360 g of permissive diet PG to make diet S. Fraction P was mixed with 360 g of diet PG to make diet P. Both diets (P and S) were sterilized and fed to mice as previously described, and both proved to be permissive (Table 4). The inhibitory effect of soybean meal could not be recovered either with the supernatant fraction (diet S) or the pellet fraction (diet P).

A third diet (diet SP) was then prepared that consisted of 360 g of diet PG mixed with both fraction S and fraction P obtained from 40 g of soybean meal and pooled after the freeze-drying step. This diet also proved to have no inhibitory effect on the establishment of *Clostridium* En. in the digestive tract of mice (Table 4).

Since the inhibitory effect could not be observed with the soybean meal fractions used separately (diet S and diet P) or combined (diet SP), we suspected that the extraction treatment destroyed the inhibitory factor. A new diet (diet T) was prepared as follows: 40 g of soybean meal was stirred in 800 ml of distilled water and then freeze-dried. The freeze-dried soybean was incorporated into 360 g of diet PG.

Diet T was nonpermissive in the same way as was the diet containing untreated soybean meal (diet soybean 5; Table 4). From this experiment, we concluded that neither the water extraction step nor the freeze-drying step was involved in the disappearance of the inhibitory effect from diets S, P, and SP. The only difference in the preparation of nonpermissive diet T and permissive diet SP was the separation of the pellet and supernatant fractions. It was possible, therefore, that the inhibitory component was a complex composed of at least two elements, one being soluble in water and the other being insoluble. These elements would not be inhibitory on their own and would not reassociate in the absence of water.

A final diet (diet S'P') was prepared by a procedure similar to that for diet SP until the

TABLE 3. Effects of dietary soybean concentration on the establishment of *Clostridium* in the intestines of mice

Diet	Composition (wt %)					Establishment
	Soybean meal	Skin milk powder	Tallow	Cerelose	Minerals + vitamin mixture	
PG (permissive)	0	66	25	5	4	Yes
2	2	64	25	5	4	Yes
5	5	61	25	5	4	No
20	20	46	25	5	4	No

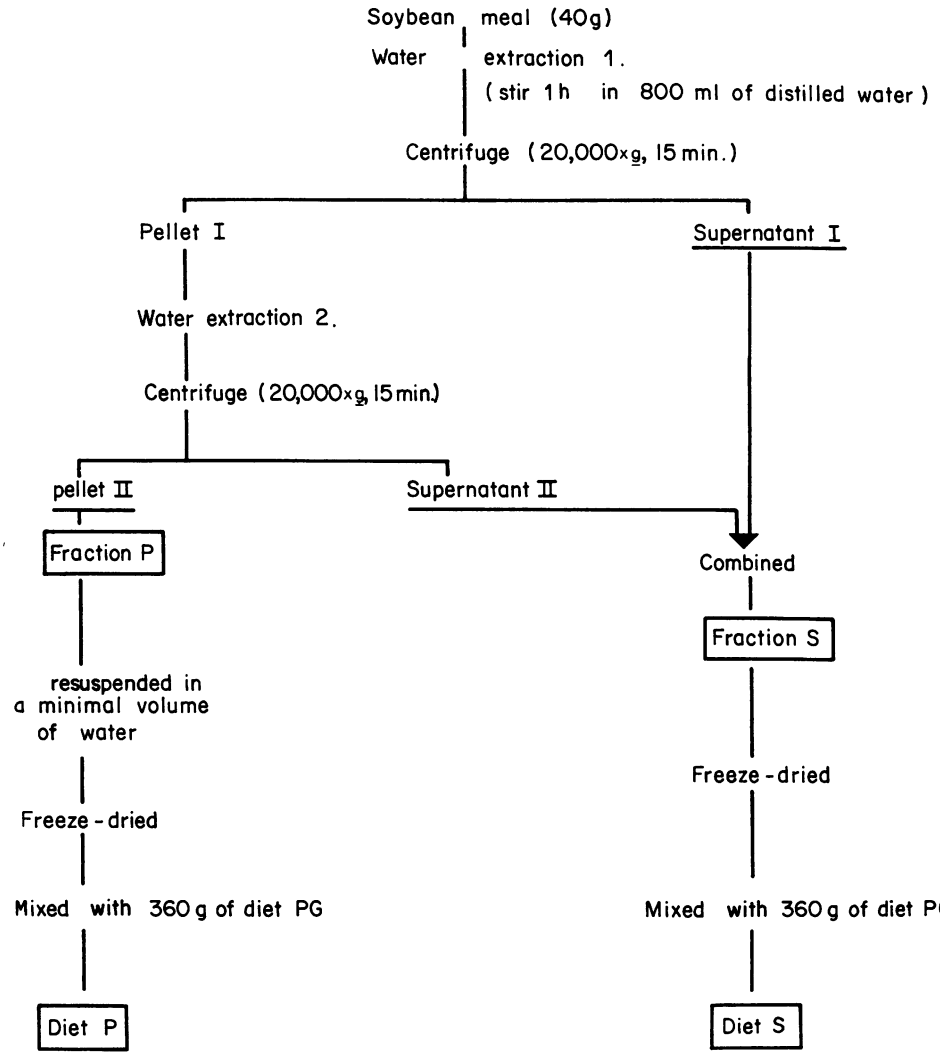

FIG. 1. Schematic representation of the fractionation procedure of soybean meal and preparation of diet S and diet P.

freeze-drying step of fractions S and P. After this step, the two fractions were mixed, suspended in a small volume of distilled water, and stirred for a few minutes. The suspension was then freeze-dried and mixed as usual with permissive diet PG. When sterilized and fed to mice, diet S'P' inhibited the establishment of *Clostridium* En. in the gastrointestinal tract of mice (Table 4). The latter result supported the hypothesis that an inhibitor composed of two components was contained in the soybean meal.

In vitro effect of soybean meal on clostridial growth. After sterilization by irradiation, about 70 g of soybean meal were transferred aseptically to a sterile vessel containing 500 ml of a nutrient broth (Schaedler liquid medium). The resulting slurry was then inoculated with 20 ml of an overnight culture of *Clostridium* En. and incubated at room temperature for a week. Examination of the culture on the 2nd day after inoculation showed a heavy growth of *Clostridium* En. We therefore concluded that soybean meal did not inhibit growth of the *Clostridium* in vitro.

Does the inhibitory element act on the germination of clostridial spores? It is known that many vegetative bacterial cells are destroyed in the stomach of animals due to the conditions of low pH and aerobiosis they encounter at that level (17). To explain the observed discrepancy between the in vivo and in vitro effects of soybean meal, it was thought that only spores of the

TABLE 4. Influence of various treatments on the inhibitory effect of soybean meal on the establishment of *Clostridium* in the intestines of mice

Diet	Treatment	Establishment of *Clostridium* En.
Soybean 5 (control)	Untreated soybean	No
S	Freeze-dried supernatant fraction	Yes
P	Freeze-dried pellet fraction	Yes
SP	Freeze-dried supernatant and pellet fractions	Yes
T	Soybean stirred in water	No
S'P'	Freeze-dried supernatant and pellet mixed in presence of water	No

Clostridium strain would enter the large bowel, where the maximal proliferation of this organism occurs. The soybean meal would perhaps inhibit spore germination in the large bowel. The following experiments were undertaken to test this hypothesis.

(i) In vivo inoculation of spores. A group of three germfree mice was fed a nonpermissive diet (soybean-containing diet U), and another group was fed the permissive diet PG. After a week of adaptation to the dietary treatment, mice were inoculated orally with a suspension of spores of *Clostridium* En. The inoculum was obtained through heating a culture at 70°C for 10 min.

Three and ten days after inoculation, feces from both groups of mice were sampled. For each sample, spores and vegetative cells were enumerated. To enumerate spores, the 10^{-2} dilution of feces was heated at 70°C for 10 min and processed as usual. Counts were obtained after 48 h of incubation for the vegetative cells and at least 5 days of incubation for the spores. *Clostridium* En. did not establish in the gastrointestinal tract of mice when they were fed the nonpermissive diet but colonized the tract when mice were fed the permissive diet (Table 5). We concluded that the presence of vegetative cells in the inoculum was not necessary for the establishment of the strain in the gastrointestinal tract.

(ii) In vitro inoculation of spores. As in the above experiment, a group of three germfree mice was fed diet U, and another group was fed diet PG. After a week of dietary treatment, the six mice were killed by cervical dislocation, and their ceca were placed separately in small tubes. Each cecum was inoculated with 10^2 spores.

A few drops of distilled water were also added to prevent desiccation. The six ceca were then incubated at 37°C. After 1 day of incubation, the spore and vegetative cells were enumerated in two ceca for each dietary treatment. The remaining two ceca (one for each diet) were examined after 2 days of incubation. The results (Table 6) indicated that there was no difference in the in vitro germination of a spore suspension under the two dietary conditions.

DISCUSSION

The first series of experiments led to the conclusion that the active fraction of the nonpermissive diets is the soybean meal and that this dietary component exerts a negative effect on the establishment of a *Clostridium* strain in the gastrointestinal tract of mice. In other words, any diet containing at least 5% soybean meal is inhibitory for the establishment of the strain (nonpermissive diet), and any diet containing less than 5% soybean meal is permissive. These unequivocal results clearly emphasize the important role of diet as a regulatory factor in the development of the gastrointestinal microflora.

Further experiments aimed at characterizing the inhibitor contained in soybean meal have shown that it is constituted of at least two components which, separately, are not inhibitory. Though the exact nature of the inhibitory fractions of soybean meal could not be determined, it is clear that they are none of the known toxic constituents of raw soybean (e.g., protease inhibitors; 12), since the soybean used in this study was heated during the production of the meal.

The inhibitory effect of soybean meal in vivo cannot be reproduced in vitro since *Clostridium* En. grows in a medium containing soybean meal. This point implies that the in vivo inhibitory effect of soybean meal is not a direct one and that the host plays a role in the observed inhibition of bacterial growth.

Several hypotheses can be considered to explain the observed discrepancy between the in vivo and in vitro effects of soybean meal or, in other words, to explain the in vivo mechanism of inhibition. (i) The inhibitory effect is realized only when soybean meal has been digested by the host. The soybean meal is not inhibitory on

TABLE 5. Establishment of *Clostridium* populations in mice on different diets, using spores as inoculum

Diet	No. of vegetative cells/g of fresh feces[a]	No. of spores/g of fresh feces[a]
Nonpermissive U	$<10^2$	$<10^2$
Permissive PG	6.10^8	2.10^5

[a] Numerations were made 10 days after the inoculation.

TABLE 6. Multiplication of *Clostridium* in cecal material obtained from mice on different diets, using spores as inoculum

Diet	No. of vegetative cells/g of cecum[a]		No. of spores/g of cecum	
	24-h incubation[b]	48-h incubation[c]	24-h incubation[b]	48-h incubation[c]
Nonpermissive	4.10^6	4.10^7	1.10^3	3.10^3
U	6.10^6		2.10^3	
Permissive	7.10^6	3.10^7	2.10^4	1.10^4
PG	1.10^7		—[d]	

[a] Each cecum was inoculated with 10^2 spores.
[b] Two ceca were used for each diet.
[c] One cecum was used for each diet.
[d] Not determined.

its own, but the inhibitory component is one of the products resulting from the digestion of soybean meal. (ii) The soybean meal influences the host in some way so that the intestinal tract is no longer suitable for the growth of the *Clostridium*. This could, for example, occur through the stimulation of an intestinal secretion by the soybean meal. The inhibitory component would, in this case, be the host's secretion. The in vivo growth of *Clostridium* En. requires some special conditions which are not necessary in vitro. This could, as we have seen in experiment 5, be an obligatory germination step in the large bowel of the mice.

To understand the mechanism of inhibition, two kinds of approaches have been used: we have tried to isolate the inhibitory component from the soybean meal fraction, thinking that its nature would give information on the mechanism of inhibition. This attempt was unsuccessful, since the water extraction procedure showed that there are at least two components involved in the inhibitory effect.

A possible mechanism of the inhibitory effect was that the soybean meal prevented the germination of the bacterial spores in the large bowel of the mice. The inoculation of mice fed permissive and nonpermissive diets produced results which do not disagree with this hypothesis, since it has been shown that vegetative cells are not necessary for the establishment of the strain and that the inhibition due to soybean meal also exists when a spore inoculum is used.

The in vitro inoculation of ceca with spores did not reveal a difference in the germination of spores in relation to the diet. It was, however, not possible to conclude from the latter result that the germination of spores is not inhibited in vivo by a dietary component because many physiological parameters were changed by removing the ceca from the animals.

In conclusion, the model used in this work may be useful in studying the relation between diet and the gastrointestinal microflora. The microflora, in this model, is reduced to only one bacterial strain, and its establishment is directly related to the composition of the diet eaten by the host. Even with this simple model, difficulties are encountered at all levels: the complexity of the nature of the inhibitory fraction which is composed of two components, and the complexity of the mechanism of inhibition in which the host's physiology is probably involved.

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